Published in final edited form as:

Mol Carcinog. 2009 April ; 48(4): 309–318. doi:10.1002/mc.20512.

Checkpoint Responses to Unusual Structures Formed by DNA Repeats

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INTRODUCTION

More than two decades ago, DNA proved to exist in several conformations different from the canonical B-DNA helix *in vitro* (reviewed in [1]). Extensive research on the existence *in vivo* and the biological consequences of these alternative DNA structures (ADS) was triggered by the later realization that structure-prone sequences are hotspots of genomic instability [2]. It is now widely believed that the instability phenomena associated with structure-prone sequences such as trinucleotide repeats, DNA palindromes and certain common fragile sites involve the formation of an ADS *in vivo*. Although several experimentally supported hypotheses for ADS-mediated genomic instability have been proposed (recently reviewed in [3]), the exact mechanisms by which ADS induce localized instability phenomena like trinucleotide repeat expansions and chromosomal breakage still remain elusive.

At the same time, our understanding of cellular responses to DNA damage expanded significantly in recent years and it became clear that checkpoint responses play a crucial role in maintaining a stable genome [4,5]. For example, cancer cells are characterized by a generalized genomic instability consisting of chromosomal deletions, translocations and increased expression of common fragile sites. The widespread genomic instability typical of cancer cells is believed to result from defects in DNA damage checkpoint responses that affect *in-trans* the stability of the genome [6].

Thus an intriguing question is: How do DNA damage checkpoints respond to alternative secondary structures? Could ADS-mediated genomic instability result from an inhibition of checkpoint responses *in-cis*? Alternatively, do checkpoint mechanisms respond to ADS, but the subsequent repair processes are ineffective?

This review summarizes current evidence on checkpoint responses to ADS with a specific focus on the checkpoint activation in response to replication fork stalling and double stranded DNA breaks caused by those structures. Throughout the review, we will be using the term “DNA damage” in its broadest sense that encompasses the various molecular events able to trigger a DNA damage checkpoint response, including DSBs and stalled replication forks.

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ALTERNATIVE DNA STRUCTURES

DNA sequences that can adopt ADS are as a rule repetitive. This property results from the requirement for some form of intra-strand base-pairing for the formation of all ADS (Fig. 1).

DNA hairpins are formed by single stranded DNA (ssDNA) that can fold back onto itself and base-pair in a DNA duplex (Fig. 1). Thus, DNA-hairpins are formed by sequences that contain inverted repeats [7,8]. While originally only perfect inverted repeats were believed to form DNA hairpins, a particular group of imperfect inverted repeats, (CNG)_n trinucleotide repeats, turned out to form mismatched hairpins as well [9]. In general, hairpin structures, even those containing multiple mismatches, are energetically favorable in ssDNA as they shield most of the hydrophobic DNA bases within the duplex DNA. The thermodynamic stability of a DNA hairpin increases with the length and the GC content of its stem [9]. Inside the cell, ssDNA is normally bound by ssDNA-binding-proteins (SSB in prokaryotes, RPA - in eukaryotes). However, the intramolecular folding into a DNA hairpin occurs so rapidly that it could win over the RPA binding to the ssDNA. Furthermore, RPA binding requires ~30 nt of ssDNA, while hairpin structures can nucleate on shorter ssDNA stretches [10]. Hairpin structures are likely to form *in vivo* during processes that require extensive DNA unwinding. Most of the data point to the formation of DNA hairpins during DNA replication *in vivo*. In *E.coli*, inverted repeat instability is replication-dependent [11,12]. In yeast, the instability of CAG repeats increases in mutants affecting DNA replication [13,14] and Okazaki fragment processing [15,16]. More recently, it was shown that DNA-hairpins formed by inverted repeats stall replication fork progression in bacteria, yeast and mammalian cells [17] and are cleaved by the bacterial SbcC nuclease in a replication dependent manner [18].

DNA cruciforms are formed by inverted repeats in double stranded DNA (Fig.1) (reviewed in [19,20]). Although a cruciform structure looks like a simple juxtaposition of two DNA-hairpins, the two types of ADS are fundamentally different in terms of the thermodynamics and kinetics of their formation. Compared to a regular DNA duplex, the cruciform-like structure is much less energetically favorable, as it contains the so-called four-way junction at its base and several unpaired bases in its loop. Thus without protein assistance, cruciform extrusion can only occur in negatively supercoiled DNA, where the relaxation of negative supercoils provides the necessary energy for the process. Furthermore, since the initial nucleation step requires extensive unwinding of the central spacer, the kinetics of cruciform extrusion is very slow except for AT-rich inverted repeats [21]. The post-nucleation extrusion of DNA cruciforms occurs by branch-migration. Consequently, inverted repeats with imperfect stems such as (CNG)_n repeats cannot form DNA cruciforms as the branch migration process is blocked even by a single base mismatch [22]. The existence of DNA cruciforms *in vivo* has been proven in bacterial cells under conditions that increase negative superhelicity, such as transcription, metabolic stress and topoisomerase mutations [23-25] and more recently in yeast cells [25a].

Slipped-strand DNA is formed by direct repeats, when a repeated unit misaligns and base-pairs with the complementary strand of another repeated unit, resulting in looped-out structures [26]. The probability of slipped-strand DNA formation increases with the length of the direct repeat and decreases for imperfect repeats. If the looped-out sequence allows intra-strand base-pairing, then the loop-outs are equivalent to DNA-hairpins.

Triple-helical H-DNA is formed by polypurine/polypyrimidine mirror repeats (reviewed in [27]). As shown in Fig.1, a DNA strand pairs with the segment of the corresponding DNA duplex, forming a triplex. This third strand comes from one half of the mirror repeat, and its complement remains single stranded. Intramolecular triplexes, similarly to cruciform

structures, are favored by negative supercoiling. Depending on whether the third strand is a polypurine or a polypyrimidine tract, the triplex is designated H-r or H-y, respectively. The H-y form requires an acidic pH, while the H-r form is stable at physiological pH in the presence of divalent cations. The presence of H-DNA *in vivo* has been probed using H-DNA-specific antibodies and triplex-forming oligonucleotides [28,29]. Recently, a particular attention was paid to the (GAA)_n repeats, expansions of which are responsible for Friedreich's ataxia[30]. This homopurine-homopyrimidine mirror repeat can form H-DNA (reviewed in [31]). Unexpectedly, when two long (GAA)_n repeats are present in the same negatively supercoiled DNA molecule, they can form a peculiar triplex structure called "sticky DNA", in which one such repeat remains a duplex, while the other GAA repeat donates its homopurine strand to form a triplex structure.

Quadruplex DNA is a tetrahelical structure formed by single-stranded DNA containing tandemly arranged runs of guanines, with a building block called the G-quartet (reviewed in [32,33]). This G-quartet is a cyclic arrangement of four guanine bases forming two Hoogsteen hydrogen bonds with each other. Monovalent cations such as sodium or potassium fit nicely into a pocket at the center of the quartet, stabilizing it at physiological conditions. Quadruplex DNA are structurally very polymorphous. They can be formed through the association of four, two or just one DNA strands. Furthermore the relative alignment of adjacent DNA strands can be parallel, alternating parallel or adjacent antiparallel. *In vitro*, G-quadruplexes are formed at physiological pH by sequences containing G-runs, including telomeric repeats [34] and CGG repeats associated with the fragile X syndrome [35].

Z-DNA is a left-handed helix formed by sequences with alternating purine and pyrimidine bases, such as (CG)_n or (CA)_n repeats (reviewed in [36]). In this DNA structure, the sugar pucker changes from C2'-endo to C3'-endo, and the configuration of the glycosidic bond changes from *anti* to *syn* between the pyrimidines and purines. These changes result in the characteristic zig-zag sugar-phosphate backbone, hence the name Z-DNA. Z-DNA has only one deep and narrow groove, corresponding to the minor groove in B-DNA, and it contains 12 bp per a left-handed helical turn. In linear DNA, Z-conformation only exists in high-salt solutions, but it is easily formed under physiological conditions in negatively supercoiled DNA. Z-DNA is also stabilized by cytosine methylation, spermine and spermidine. Z-DNA-forming sequences are abundant in the human genome, occurring as frequently as once in 3000 bp [37]. Not surprisingly therefore, antibodies against Z-DNA interact with multiple sites at active eukaryotic genes. It was suggested that *in vivo*, Z-DNA is primarily formed during transcription. An advancing RNA polymerase generates a wave of negative supercoiling behind, and thus favors Z-DNA formation in the negatively supercoiled DNA.

SECONDARY STRUCTURE-MEDIATED GENOMIC INSTABILITY

DNA sequences that can adopt ADS have been shown to undergo a wide variety of genomic instability phenomena both in humans and in model organisms. Virtually all sequences that can form stable ADS have been associated *in vivo* with chromosomal deletions, breaks or gross chromosomal rearrangements [38]. This suggests that the instability phenomena are not driven by the primary DNA sequence but rather by the existence of an ADS.

Expansions and contractions of tandem repeats

Based on the size of the repeated unit, tandem repeats are classified in microsatellites (1-10 bp), minisatellites (10 – 100 bp) and satellites (above 100 bp). From the point of view of human pathology, one of the most significant classes of tandem repeats are trinucleotide repeats (TNRs), a subtype of microsatellites. More than a dozen human hereditary neurodegenerative disorders are caused by expansions of TNRs [30]. Expansions of CAG

repeats cause Huntington's disease, myotonic dystrophy and several types of spinocerebellar ataxias. The fragile X (FX) syndrome, the FRAXE mental retardation and FX-associated tremor and ataxia syndrome are caused by expanded CGG repeats. In addition, expanded CGG tracts have been mapped at all the human folate-sensitive fragile sites (reviewed in [39]). Friedreich's ataxia, the most frequent form of inherited ataxia, is caused by an expanded GAA repeat [30].

In normal individuals, TNRs are stably transmitted. A normal-length TNR may convert to a pre-mutation allele, often by loss of stabilizing interruptions [30]. Pre-mutation TNRs do not cause a disease phenotype but are prone to a phenomenon called dynamic mutation: the repeat undergoes further expansions during intergenerational transmission, and the probability of repeat expansion increases with the repeat length. Once the TNR tract reaches the full mutation length, the disease occurs. The full-mutation TNR length, as well as the mechanism by which the full-mutation repeat leads to the disease, depend on: (a) the type of repeat, (b) the repeat's location within the gene, and (c) the particular gene affected. On the other hand, the mechanism that cause repeat expansions seems to have common features for various TNRs [30]. An early observation regarding TNR expansions was that only those repeated sequences that could form an ADS expanded in humans [9]. In model organisms TNRs undergo several types of instability [40]. In both bacteria and yeast the predominant instability phenomena observed are repeat contractions. Repeat expansions also occur, although at a much lower rate.

In addition to TNR expansions, expansions of tetra-, penta- and dodecanucleotide repeats have been recently associated with hereditary neurological disorders [30]. Microsatellite instability also occurs in certain cancers, characterized by mutations in the DNA mismatch repair machinery. This type of instability frequently involves CA dinucleotide repeats. However, unlike TNR expansions, which occur at a single locus and are fairly large (often several times the initial repeat length), dinucleotide repeats instability due to mismatch repair defects occurs throughout the genome and involves small-size deletions or expansions.

Chromosomal breakage and gross chromosomal rearrangements

Inverted repeats, Z-DNA and H-DNA-forming sequences, as well as trinucleotide repeats have all been shown to represent hotspots of chromosomal breaks, homologous recombination and gross chromosomal rearrangements in prokaryotic and eukaryotic cells [41,42].

In humans, long AT-rich inverted repeats have been mapped at sites of recurrent hereditary translocations: t(11,22), t(17,22) [43]. It was proposed that formation of a large, AT-rich cruciform could be a substrate for DNA cleavage, which, in turn, can result in the translocation [44].

Z-DNA-forming sequences were found near chromosomal breakpoints involving the *c-MYC* and *BCL-2* genes [42]. In addition, sequences prone to adopt Z-DNA conformations induce DSBs and consequent large deletions in mammalian cells [45].

The *c-MYC* promoter contains a H-DNA-forming sequence that induces DSBs in mammalian cells. The repair of double stranded breaks (DSBs) resulted in mutations in the areas adjacent to the H-DNA-forming sequence, showing that H-DNA can be mutagenic [46]. A H-DNA-prone polypurine/polypyrimidine repeat is also present in an intron of *PKD1*, the gene mutated in the autosomal dominant polycystic kidney disease. It was proposed that formation of H-DNA could be responsible for the unusually high mutation rates in the *PKD1* gene, which translates into disease [47]. An ADS-forming sequence, possibly

forming DNA triplex, was mapped at the breakpoint site of t(14,18), a translocation that frequently occurs in human lymphomas. The initial breakage event that leads to t(14,18) appears to be a DSB induced by the RAG2 nuclease in a ADS-specific manner [48].

In summary, ADS turn out to be particularly vulnerable spots of the genome, and thus it becomes important to understand how genome surveillance mechanisms, such as DNA damage checkpoint responses, function at these sites.

BRIEF OVERVIEW OF DNA DAMAGE CHECKPOINT RESPONSES

The DNA damage response is a signal-transduction cascade that is initiated by several types of DNA damage and leads to cell cycle arrest, activation of DNA repair mechanisms and in certain cases apoptosis. When DNA damage occurs in the form of stalled replication forks, the damage response includes inhibition of origin firing and stabilization of stalled replisomes. Depending on the cell cycle phase when the DNA damage and the cell cycle arrest occur, the damage response is subdivided into a G1/S, an intra-S and a G2/M checkpoint response.

For simplicity, the DNA damage cascade can be conceived as composed of two major branches: one that responds primarily to double stranded breaks (DSBs) and is dependent on the mammalian ATM kinase (*S. cerevisiae* Tel1) (recently reviewed in [49]) and another one that mainly responds to stalled replication forks and DNA gaps and its major player in mammals is the ATR kinase (*S. cerevisiae* Mec1) (reviewed in [50]). However, the two pathways are interconnected, not only because the triggering structures (DSBs, gaps and stalled replication forks) are inter-convertible, but also because the proteins involved in the two cascades cross-regulate each-other.

The initial signal that activates the ATR pathway (Fig. 2a) consists of ssDNA bound by RPA and an ssDNA-dsDNA junction [50]. This structure independently recruits two components of the recognition complex: ATR, that binds to the ssDNA-RPA complex[51] and the clamp loader-like complex Rad17-RFC (*S. cerevisiae* Rad24-RFC). Rad17-RFC loads the processivity clamp-like complex, 9-1-1 composed of Rad9, Rad1 and Hus1 (*S. cerevisiae* Rad17, Mec3, Ddc1) that recognizes the ssDNA-dsDNA junction [52]. In yeast, 9-1-1 directly activates Mec1[53], while in mammals 9-1-1 needs to recruit TopBP1 for appropriate ATR activation [54].

After the initial signaling step, mediators play the role of bringing effector kinases - mammalian CHK1 and *S. cerevisiae* Rad53 - in the proximity of ATR. Once activated by ATR, effector kinases phosphorylate several downstream targets, ultimately leading to cell cycle arrest, inhibition of further replication origin firing, transcriptional activation of DNA repair genes and stabilization of stalled replisomes.

Claspin (*S. cerevisiae* Mrc1) is the mediator of checkpoint responses during replication stress [55]. In yeast, checkpoint responses to other types of DNA damage are mediated by Rad9. The human homologue of Rad9 is the p53 binding protein 53BP1, but BRCA1 is also implicated in activating CHK1 in response to DNA damage [56].

Recent studies have dissected out the roles of Mrc1 at stalled replication forks. Mrc1 is a highly charged protein that contains several SQ/TQ residues that are phosphorylated in a Mec1 dependent manner [55]. Rad53 binds phosphorylated Mrc1 and this leads to Mec1-dependent phosphorylation of Rad53 as well as Rad53 auto-phosphorylation, resulting in Rad53 activation and signal amplification [57]. Activated Rad53 phosphorylates several downstream targets, including replisome components, leading to an inhibition of late replication origin firing and stabilization of stalled replisomes. The Mec1/Rad53 dependent

mechanisms of preventing the collapse of stalled replisomes are not entirely understood, but are believed to involve phosphorylation of RPA, PCNA and DNA polymerase alpha [58].

In addition to its mediator function, Mrc1 proved to have a checkpoint-independent function in stabilizing stalled replication forks. Mrc1 forms a complex with Tof1 (mammalian Timeless) and Csm3 (mammalian Tipin) that travels with the replication fork [59]. The physical presence of this complex at stalled forks is believed to maintain replisome integrity. In the absence of either Mrc1 or Tof1, replisomes stalled by hydroxyurea dissociate from the site of DNA synthesis [59]. The rate of DNA replication under normal conditions is reduced in both Mrc1 and Tof1 deletion strains, although the replication defect is more severe for the Mrc1 mutant [60,61]. Notably, a checkpoint deficient allele of Mrc1 (Mrc1AQ), that has mutated SQ/TQ phosphorylation sites, is competent in maintaining a normal replication pattern [57], thus allowing the separation of checkpoint-dependent and checkpoint-independent functions of Mrc1. Reduced replication speed was also described in the absence of either Claspin or Timeless in mammalian cells [62,63]. In addition, in human cells Claspin and Timeless have an ATR-independent role in triggering PCNA ubiquitination (which is required for loading trans-lesion polymerases) following hydroxyurea or UV exposure [64].

While the ATR pathway has a basal level of activation during S-phase, presumably due to occasional fork stalling during normal replication, the ATM pathway is rarely but rapidly activated in response to DSBs (reviewed in [49]). DSBs are recognized by the MRN: Mre11-Rad50-Nbs1 (*S. cerevisiae* MRX: Mre11-Rad50-Xrs2) complex that in turn recruits the ATM kinase. ATM-mediated phosphorylation of H2AX histone tails leads to the recruitment of MDC1 (mediator of damage checkpoint) through its BRCT domain. MDC1 recruits additional ATM kinase, leading to signal amplification and spreading of γ H2AX along the chromatin. Activated ATM also phosphorylates CHK2, the effector kinase of the DSB response cascade. In yeast, both Mec1 and Tel1 transduce signals through the CHK2 homologue, Rad53, while Tel1 also activates *S. cerevisiae* Chk1.

THE INTERPLAY BETWEEN DNA DAMAGE CHECKPOINT RESPONSES AND ALTERNATIVE SECONDARY STRUCTURES

To begin rationalizing the interplay between ADS and DNA damage checkpoint responses we will consider the following questions:

1. Are ADS *per se* recognized as a form of DNA damage?
2. Does the presence of an ADS induce DNA damage by interfering with DNA replication or DNA repair?
3. If so, do the structures generated at damaged sites within an ADS resemble the damaged DNA in the regular B-DNA helix closely enough to be properly recognized by the checkpoint machinery?

The structural features of ADS that distinguish them from the B-DNA helix and thus could be recognized as damaged DNA are the presence of unpaired bases and the helix distortions/junctions. However the amount of ssDNA required to trigger a checkpoint response normally exceeds 300 bp [58], making short loops of ssDNA present in ADS unlikely triggers of checkpoint activation. The helix distortion that occurs with imperfect hairpin structures at CAG repeats is recognized by the mismatch repair machinery [65]. The Msh2/Msh3 complex specifically binds CAG-hairpins and this interaction alters the ATP-ase activity of the complex.

While ADS *per se* are probably an infrequent trigger of DNA damage checkpoint responses, and, thus, should not be regarded as a form of DNA damage, there is extensive evidence suggesting that ADS can induce checkpoint-triggering events such as DSBs and replication fork stalling.

Checkpoint Responses to Replication Fork Stalling at ADS

DNA repeats that can adopt ADS are known to inhibit DNA polymerization *in vitro* [66,67]. Because of the tight control of intracellular ssDNA and the requirement of specific, non-physiological ionic conditions for the formation of certain ADS, it was not immediately clear whether ADS also occur within the cell and if so, whether or not they would be stable enough to prevent replisome progression. A significant body of evidence however, has been accumulated in recent years, supporting the concept that ADS are natural replication pause sites: two-dimensional electrophoresis experiments showed that CGG repeats transiently stall replication fork progression in bacteria [68] as well as in yeast [69] and mammalian cells [70]; similarly, long inverted repeats arrest replisome progression in bacteria, yeast and mammals by forming DNA hairpins [17]; a sequence present at the human common fragile site FRA16D that includes a potentially ADS-forming AT repeat blocks replication in yeast (Zhang and Freudenreich, 2008); triplex-forming GAA repeats arrest replication fork progression and induce chromosomal fragility in yeast [71], and attenuate replication in mammalian cells (M.Leffak personal communication).

Widespread replication stalling caused by replication inhibition with hydroxyurea, that depletes nucleotide pools, or by DNA damage following UV irradiation, triggers a checkpoint response that leads to replication fork stabilization, inhibition of late-origin firing and cell cycle arrest (see above). Under hydroxyurea treatment, long stretches of ssDNA (~300 bp) are exposed [72], and this is believed to occur as a result of uncoupling between the helicase and the arrested polymerase. The long stretch of ssDNA bound by RPA triggers checkpoint activation ([51,73]) (Fig 2a). Other types of replication fork stalling, when both the helicase and the polymerase are arrested, and thus not much ssDNA is generated, appear to elude the checkpoint activation. Interstrand crosslinks for example induce minor checkpoint activation in fission yeast [74] while replication blockage caused by protein-DNA complexes do not trigger a checkpoint response [75].

Replication fork arrest at DNA-hairpins, that occurs in eukaryotes at CGG repeats and inverted repeats, shares certain features with both of the above scenarios: it could allow uncoupling of the helicase and polymerase, but would not necessarily expose ssDNA. The formation of DNA hairpins requires the repeat to be in a single stranded state. Thus, DNA hairpins could be formed during the lagging strand synthesis, when a single stranded region of about 200bp is required for Okazaki fragment priming [76]. The DNA-hairpin formed on the lagging template, behind the advancing helicase, would block the lagging strand polymerase. *In-vitro* experiments showed that replication stalling at a damaged site on the lagging strand template does not cause persistent stalling since the helicase and leading polymerase progress further, while the lagging polymerase quickly re-starts at the next Okazaki fragment [77,78]. However, in the case of CGG and inverted repeats replication intermediates accumulate at the repeat showing a clear replication fork arrest [17,69]. Presumably, this is due to the fact that the repeated template ahead of the fork, does not allow efficient re-priming of the lagging template.

Regardless of the mechanism of lagging strand polymerase arrest, as the progression of the helicase is not impeded, there would be uncoupling between the helicase and the polymerase. Notably, the ssDNA exposed on the repetitive template during uncoupling can fold into a hairpin-like structure that would not bind RPA and thus might not trigger a checkpoint response. Consistent with this hypothesis, recent evidence show that the

replication fork arrest at CGG repeats in yeast is not affected in the absence of Mrc1's checkpoint function. However, it is increased in an Mrc1 deletion strain [70]. Therefore, it appears that the replication fork stabilization at CGG hairpins requires the physical presence of the Mrc1 protein at the fork, rather than its checkpoint function, suggesting that replication stalling at CGG repeats does not activate the replication checkpoint (Fig. 2b, left). It is worth mentioning that it is not currently known whether replication fork stalling at a single DNA damage site, either on the leading or on the lagging strand, triggers a checkpoint response. Given that eukaryotic chromosomes have multiple origins of replication, it is conceivable that a single stalled fork is not deleterious enough to warrant checkpoint activation. Thus replication fork stalling at ADS not only generates very little ssDNA but is also localized (as opposed to the widespread fork arrest under hydroxyurea or UV treatments), making it a poor activator of replication checkpoint responses.

The checkpoint triggering potential of stalled replication forks depends not only on the configuration of the stalled intermediate but also on the way it is further processed. Upon stalling, replication forks can regress in order to undergo recombinational restart [79]. Replication forks progressing through CAG repeats frequently regress *in vitro* [80]. The reversed fork contains a double-stranded end potentially recognized by the MRN (*S. cerevisiae* MRX) complex. In addition, reversed forks are subject to nuclease processing, that generates a stretch of ssDNA [81]. Recent evidence indicates that replication forks progressing through CAG repeats on a yeast chromosome also undergo fork regression in [82]. The ssDNA exposed following end-resection at a regressed fork could trigger checkpoint activation (Fig. 2b, right), explaining the fact that chromosomal breakage at CAG repeats is increased in the absence of cellular checkpoint function (see below).

Checkpoint Responses at ADS-forming Chromosomal Fragile Sites

Virtually every structure-forming repeat that causes replication fork stalling *in vivo* was also shown to induce double stranded breaks (DSBs) in model organisms. As mentioned above, expanded CGG repeats are causative for folate-sensitive fragile sites in humans. Although it is not known whether the DNA duplex actually breaks at fragile sites in human cells, it was shown that DSBs occur at CGG repeats when they are inserted into a yeast chromosome [83]. Chromosomal breaks also occur at CAG and GAA repeats in yeast [16,84]. The CAG repeat fragility increases in cells lacking the checkpoint protein Mec1 or containing a checkpoint deficient allele of Rad53 [85] suggesting that the DNA damage that occurs at CAG repeats is sensed by the checkpoint machinery. The initial form of DNA damage could be a DSB *per se* or a different type of damage such as a nick, a gap or stalled replication forks that could be eventually converted to a double stranded break. Interestingly, the effect of checkpoint adaptor proteins Mrc1 and Rad9 on CAG fragility differed depending on the CAG tract length [86]. The increase in fragility at the CAG repeat in a checkpoint defective Mrc1 mutant (Mrc1-1) compared to the wild type was significantly more pronounced for 155 CAG repeats than for 85 repeats, indicating that sensing of stalled forks is more important for preventing fragility of the longer repeat. These results suggested that DSBs at the long repeat occur primarily by a mechanism involving stalled replication forks. Recent results comparing the effect of a deletion of Mrc1 to Mrc1 checkpoint-deficient alleles show that CAG repeat fragility is even more pronounced in the complete absence of the Mrc1 protein, a situation where fork uncoupling occurs (Gellon, L. Lahiri, M. and Freudenreich C., in preparation). Therefore both the fork stabilization and checkpoint signaling functions of the Mrc1 protein are critical for preventing chromosome breakage at ADS-forming CAG repeats.

Interestingly, for GAA repeats chromosomal fragility is due to the cleavage of a triplex by the mismatch repair machinery [84]. Similarly, Z-DNA forming sequences induce chromosomal breaks and large deletions in mammalian cells, and this phenomenon appears

to result from cleavage of the Z-DNA structure and to be partially independent of DNA replication [45]. The DNA damage checkpoint activation at Z-DNA and H-DNA induced chromosomal breaks has not yet been addressed. Thus future experiments addressing this question might bring interesting evidence regarding checkpoint responses at DSBs at an ADS site, and could clarify whether the dynamics of checkpoint responses differ following enzymatic cleavage of different ADS.

Long inverted repeats that can form stable hairpin and cruciform structures were proposed to be an equivalent of fragile sites in yeast [87]. They induce hairpin-capped DSBs and the occurrence of breakage is more frequent under low levels of DNA polymerase alpha. The symmetric, hairpin-capped structure of the breakage intermediate suggested that it occurs as a result of the cleavage of a cruciform [88]. Alternatively, hairpin intermediates formed during replication could be cleaved on either side of the hairpin, leading to the same type of breakage products. A similar phenomenon is observed in *E.coli*, where IRs are cleaved by the SbcCD nuclease leading to symmetric, hairpin-capped DSB intermediates [18]. In the bacterial case, it was shown that the nuclease cleavage is replication dependent and occurs after the replication fork passes the repeat. Thus it was proposed that hairpin structures formed during replication, rather than cruciforms are the substrate of SbcCD. Consistent with this observation, replication fork stalling at long IRs is caused by hairpin structures both in yeast and mammalian cells [17]. Taken together, the above data support the idea that long inverted repeats form hairpin like structures during lagging strand replication which in turn transiently stall replication fork progression. The break-down of stalled forks or the nuclease-mediated cleavage of hairpin structures left behind the fork are responsible for the hairpin-capped DSBs. However, the eukaryotic nuclease responsible for hairpin cleavage remains thus far a mystery. The components of the MRX complex as well as the Sae2 nuclease are required for the repair of hairpin-capped DSBs [88]. In the absence of the above proteins, hairpin-capped DSBs accumulate and the rate of homologous recombination events at the inverted repeat is reduced. It is not obvious whether the hairpin-capped DSBs are equivalent to the "open-ended" DSBs in terms of recruiting the MRX complex and triggering a checkpoint response, but the above results suggest that MRX can recognize and process hairpin-capped DSBs. Chromosomal sequences that are prone to increased breakage in yeast in the absence of either Mec1 or Rad53 include inverted Ty repeats, [89]. This observation further supports the concept that although they have a non-canonical end-structure, DSBs that occur at inverted repeats trigger a checkpoint response that is important for the break repair.

In summary, the experimental evidence currently available, although far from providing a complete picture of the mechanisms of checkpoint activation at an ADS suggest that: (a) ADS are potential triggers of DNA damage checkpoint responses mainly by inducing replication fork stalling and chromosomal breaks, (b) ADS generate specific DNA conformations at the damaged site, that may influence the checkpoint signaling, and (c) the dynamics of checkpoint activation are likely to differ at different types of ADS.

Checkpoint Responses and Trinucleotide Repeat Instability

The molecular mechanisms of trinucleotide repeat (TNR) instability in humans and in model organisms are still under investigation, but the large amount of data accumulated so far support a causative role for DNA replication, as well as DNA repair recombination, and transcription through the repeat. Recent data indicate that checkpoint proteins are important in preventing TNR instability.

Yeast cells containing checkpoint-deficient alleles of Mrc1 or Rad53 had a significant increase in contractions of a CAG-70 tract, as well as an increase in expansions [85,86]. In addition, deletion of Mec1, Ddc2, Rad9, Chk1, or any of the components of the yeast 9-1-1

complex led to increases in contractions, and in some cases also increased expansions. More recent results using shorter CAG repeats (13-20 repeats) confirm that DNA damage checkpoint is important for inhibiting expansions after formation of repeat-dependent structures [90]. As mentioned above, the DNA damage checkpoint is not only important in regulating cell cycle events, but also in mediating appropriate repair and fork restart processes. These processes appear to occur with less fidelity in the absence of a fully functional checkpoint response, leading to repeat instability. Further investigation of the fork stabilization role of the Mrc1/Tof1/Csm3 complex indicates that this complex is especially important in preventing repeat contractions ([90], Gellon, L. Lahiri, M. and Freudenreich C., in preparation). Uncoupling of the polymerase from the replicative helicase has been shown to increase single-stranded template DNA [78] and spontaneous structure formation on this ssDNA is the precursor to a contraction event. Thus, an intact structure of the fork traversing ADS is especially important to prevent genome instability. Altogether, these results indicate that the DNA damage checkpoint response responds to some types of ADS's, such as expanded CAG/CTG repeats, and is an important component of maintaining genome stability at these sequences.

A similar conclusion was reached by a study using ATR +/- heterozygous mice carrying a CGG repeat at the mouse *Fmr1* locus. In these mice CGG repeats showed increased expansion frequency during intergenerational transmission as compared to wild-type mice. CGG repeat expansions also occurred with increased frequency in somatic cells in ATR +/- mice [91]. The occurrence of ATR-sensitive somatic expansions in post-mitotic cells such as adult brain tissue, suggests that replication-independent DNA damage at CGG repeats trigger an ATR-mediated checkpoint response, that is important in preventing CGG repeat expansions

Given that no heritable mutation in the DNA damage checkpoint machinery has been associated with TNR expansion diseases, the above data indicate the possibility that TNR expansions occur as a result of DNA damage checkpoint escape at the ADS. However, more experimental evidence is needed to validate this hypothesis.

PERSPECTIVE

We propose here a model for the role of checkpoint responses at ADS in regulating chromosomal fragility at CGG and CAG repeats. While the mechanistic details of this model still need to be solved, it frames our current knowledge on the role of checkpoints in ADS-mediated genome instability.

It is now clear that ADS are hotspots of DNA damage that require appropriate checkpoint responses. In addition, the efficiency of checkpoint activation depends on the DNA structure generated at the damaged site. As a consequence, certain ADS, such as CGG-hairpins, may elude the checkpoint response (see above). The lack of an S-phase checkpoint response to replication fork stalling at CGG repeats results in cell cycle progression despite persistent unreplicated areas around the repeat, leading to chromosomal fragility (Fig. 2b, left).

On the other hand, CAG repeats although unstable, are not associated with chromosomal fragility in humans. In addition, the rates of chromosomal breakage observed at CAG repeats in yeast are much lower than for CGG repeats [16]. As mentioned above, the finding that the checkpoint functions of Rad53p and Mrc1p are important in preventing breakage at CAG repeats implies that this ADS may invoke an S-phase response. Therefore, it is possible that the checkpoint response triggered at the CAG repeat, possibly by ssDNA exposed at reversed replication forks (see above), induces appropriate repair mechanisms that prevent chromosomal breakage (Fig 2b, right).

Although unraveling the interplay between secondary DNA structures and checkpoint responses is yet as its very beginning, we could envision that the magnitude of genomic instability phenomena induced by a given ADS would be the result of two components: the potential of the ADS to induce DNA damage and its propensity to elude checkpoint responses.

Acknowledgments

This study was supported by the NIH grants GM60987 to S.M.M and GM063066 to C.F.

References

1. Mirkin SM. Discovery of alternative DNA structures: a heroic decade (1979-1989). *Front Biosci.* 2008; 13:1064–1071. [PubMed: 17981612]
2. Sinden RR, Wells RD. DNA structure, mutations, and human genetic disease. *Curr Opin Biotechnol.* 1992; 3:612–622. [PubMed: 1369117]
3. Kovtun IV, McMurray CT. Features of trinucleotide repeat instability in vivo. *Cell Res.* 2008; 18:198–213. [PubMed: 18166978]
4. Toueille M, Hubscher U. Regulation of the DNA replication fork: a way to fight genomic instability. *Chromosoma.* 2004; 113:113–125. [PubMed: 15300444]
5. Jeggo PA, Lobrich M. Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability. *DNA Repair (Amst).* 2006; 5:1192–1198. [PubMed: 16797253]
6. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature.* 2004; 432:316–323. [PubMed: 15549093]
7. Bissler JJ. DNA inverted repeats and human disease. *Front Biosci.* 1998; 3:d408–418. [PubMed: 9516381]
8. Sinden, RR. DNA structure and function. Academic Press, Inc; San Diego: 1994.
9. Gacy AM, Goellner G, Juranic N, Macura S, McMurray CT. Trinucleotide repeats that expand in human disease form hairpin structures in vitro. *Cell.* 1995; 81:533–540. [PubMed: 7758107]
10. Nag DK, Petes TD. Seven-base-pair inverted repeats in DNA form stable hairpins in vivo in *Saccharomyces cerevisiae*. *Genetics.* 1991; 129:669–673. [PubMed: 1752412]
11. Lindsey JC, Leach DR. Slow replication of palindrome-containing DNA. *J Mol Biol.* 1989; 206:779–782. [PubMed: 2738918]
12. Shurvinton CE, Stahl MM, Stahl FW. Large palindromes in the lambda phage genome are preserved in a rec+ host by inhibiting lambda DNA replication. *Proc Natl Acad Sci U S A.* 1987; 84:1624–1628. [PubMed: 2951734]
13. Callahan JL, Andrews KJ, Zakian VA, Freudenreich CH. Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. *Mol Cell Biol.* 2003; 23:7849–7860. [PubMed: 14560028]
14. Schweitzer JK, Livingston DM. The effect of DNA replication mutations on CAG tract stability in yeast. *Genetics.* 1999; 152:953–963. [PubMed: 10388815]
15. Schweitzer JK, Livingston DM. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. *Hum Mol Genet.* 1998; 7:69–74. [PubMed: 9384605]
16. Freudenreich CH, Kantrow SM, Zakian VA. Expansion and length-dependent fragility of CTG repeats in yeast. *Science.* 1998; 279:853–856. [PubMed: 9452383]
17. Voineagu I, Narayanan V, Lobachev KS, Mirkin SM. Replication stalling at unstable inverted repeats: interplay between DNA hairpins and fork stabilizing proteins. *Proc Natl Acad Sci U S A.* 2008; 105:9936–9941. [PubMed: 18632578]
18. Eykelenboom JK, Blackwood JK, Okely E, Leach DR. SbcCD causes a double-strand break at a DNA palindrome in the *Escherichia coli* chromosome. *Mol Cell.* 2008; 29:644–651. [PubMed: 18342610]
19. Leach DR. Long DNA palindromes, cruciform structures, genetic instability and secondary structure repair. *Bioessays.* 1994; 16:893–900. [PubMed: 7840768]

20. Lewis SM, Cote AG. Palindromes and genomic stress fractures: bracing and repairing the damage. *DNA Repair (Amst)*. 2006; 5:1146–1160. [PubMed: 16807136]
21. Zheng GX, Sinden RR. Effect of base composition at the center of inverted repeated DNA sequences on cruciform transitions in DNA. *J Biol Chem*. 1988; 263:5356–5361. [PubMed: 3356690]
22. Panyutin IG, Hsieh P. Formation of a single base mismatch impedes spontaneous DNA branch migration. *J Mol Biol*. 1993; 230:413–424. [PubMed: 8464057]
23. Zheng GX, Kochel T, Hoepfner RW, Timmons SE, Sinden RR. Torsionally tuned cruciform and Z-DNA probes for measuring unrestrained supercoiling at specific sites in DNA of living cells. *J Mol Biol*. 1991; 221:107–122. [PubMed: 1920399]
24. Krasilnikov AS, Podtelezhnikov A, Vologodskii A, Mirkin SM. Large-scale effects of transcriptional DNA supercoiling in vivo. *J Mol Biol*. 1999; 292:1149–1160. [PubMed: 10512709]
25. Dayn A, Malkhosyan S, Mirkin SM. Transcriptionally driven cruciform formation in vivo. *Nucleic Acids Res*. 1992; 20:5991–5997. [PubMed: 1461732]
- 25a. Coté AG, Lewis SM. Mus81-dependent double-strand DNA breaks at in vivo-generated cruciform structures in *S. cerevisiae*. *Mol Cell*. 2008; 31:800–812. [PubMed: 18922464]
26. Sinden RR, Pytlos-Sinden MJ, Potaman VN. Slipped strand DNA structures. *Front Biosci*. 2007; 12:4788–4799. [PubMed: 17569609]
27. Frank-Kamenetskii MD, Mirkin SM. Triplex DNA structures. *Annu Rev Biochem*. 1995; 64:65–95. [PubMed: 7574496]
28. Agazie YM, Burkholder GD, Lee JS. Triplex DNA in the nucleus: direct binding of triplex-specific antibodies and their effect on transcription, replication and cell growth. *Biochem J*. 1996; 316(Pt 2):461–466. [PubMed: 8687388]
29. Ohno M, Fukagawa T, Lee JS, Ikemura T. Triplex-forming DNAs in the human interphase nucleus visualized in situ by polypurine/polypyrimidine DNA probes and antitriplex antibodies. *Chromosoma*. 2002; 111:201–213. [PubMed: 12355210]
30. Mirkin SM. Expandable DNA repeats and human disease. *Nature*. 2007; 447:932–940. [PubMed: 17581576]
31. Wells RD. DNA triplexes and Friedreich ataxia. *Faseb J*. 2008; 22:1625–1634. [PubMed: 18211957]
32. Maizels N. Dynamic roles for G4 DNA in the biology of eukaryotic cells. *Nat Struct Mol Biol*. 2006; 13:1055–1059. [PubMed: 17146462]
33. Huppert JL. Thermodynamic prediction of RNA-DNA duplex-forming regions in the human genome. *Mol Biosyst*. 2008; 4:686–691. [PubMed: 18493667]
34. Williamson JR, Raghuraman MK, Cech TR. Monovalent cation-induced structure of telomeric DNA: the G-quartet model. *Cell*. 1989; 59:871–880. [PubMed: 2590943]
35. Fry M, Loeb LA. The fragile X syndrome d(CGG)n nucleotide repeats form a stable tetrahelical structure. *Proc Natl Acad Sci U S A*. 1994; 91:4950–4954. [PubMed: 8197163]
36. Rich A, Zhang S. Timeline: Z-DNA: the long road to biological function. *Nat Rev Genet*. 2003; 4:566–572. [PubMed: 12838348]
37. Hamada H, Kakunaga T. Potential Z-DNA forming sequences are highly dispersed in the human genome. *Nature*. 1982; 298:396–398. [PubMed: 6283389]
38. Bacolla A, Wojciechowska M, Kosmider B, Larson JE, Wells RD. The involvement of non-B DNA structures in gross chromosomal rearrangements. *DNA Repair (Amst)*. 2006; 5:1161–1170. [PubMed: 16807140]
39. Lukusa T, Fryns JP. Human chromosome fragility. *Biochim Biophys Acta*. 2008; 1779:3–16. [PubMed: 18078840]
40. Lenzmeier BA, Freudenreich CH. Trinucleotide repeat instability: a hairpin curve at the crossroads of replication, recombination, and repair. *Cytogenet Genome Res*. 2003; 100:7–24. [PubMed: 14526162]
41. Lobachev KS, Rattray A, Narayanan V. Hairpin- and cruciform-mediated chromosome breakage: causes and consequences in eukaryotic cells. *Front Biosci*. 2007; 12:4208–4220. [PubMed: 17485368]

42. Wang G, Vasquez KM. Non-B DNA structure-induced genetic instability. *Mutat Res.* 2006; 598:103–119. [PubMed: 16516932]
43. Kurahashi H, Inagaki H, Ohye T, Kogo H, Kato T, Emanuel BS. Palindrome-mediated chromosomal translocations in humans. *DNA Repair (Amst).* 2006; 5:1136–1145. [PubMed: 16829213]
44. Kogo H, Inagaki H, Ohye T, Kato T, Emanuel BS, Kurahashi H. Cruciform extrusion propensity of human translocation-mediating palindromic AT-rich repeats. *Nucleic Acids Res.* 2007; 35:1198–1208. [PubMed: 17264116]
45. Wang G, Christensen LA, Vasquez KM. Z-DNA-forming sequences generate large-scale deletions in mammalian cells. *Proc Natl Acad Sci U S A.* 2006; 103:2677–2682. [PubMed: 16473937]
46. Wang G, Vasquez KM. Naturally occurring H-DNA-forming sequences are mutagenic in mammalian cells. *Proc Natl Acad Sci U S A.* 2004; 101:13448–13453. [PubMed: 15342911]
47. Patel HP, Lu L, Blaszk RT, Bissler JJ. PKD1 intron 21: triplex DNA formation and effect on replication. *Nucleic Acids Res.* 2004; 32:1460–1468. [PubMed: 14990751]
48. Raghavan SC, Swanson PC, Wu X, Hsieh CL, Lieber MR. A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. *Nature.* 2004; 428:88–93. [PubMed: 14999286]
49. Lavin MF, Kozlov S. ATM activation and DNA damage response. *Cell Cycle.* 2007; 6:931–942. [PubMed: 17457059]
50. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol.* 2008; 9:616–627. [PubMed: 18594563]
51. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science.* 2003; 300:1542–1548. [PubMed: 12791985]
52. Ellison V, Stillman B. Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA. *PLoS Biol.* 2003; 1:E33. [PubMed: 14624239]
53. Majka J, Niedziela-Majka A, Burgers PM. The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint. *Mol Cell.* 2006; 24:891–901. [PubMed: 17189191]
54. Kumagai A, Lee J, Yoo HY, Dunphy WG. TopBP1 activates the ATR-ATRIP complex. *Cell.* 2006; 124:943–955. [PubMed: 16530042]
55. Alcasabas AA, Osborn AJ, Bachant J, Hu F, Werler PJ, Bousset K, Furuya K, Diffley JF, Carr AM, Elledge SJ. Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol.* 2001; 3:958–965. [PubMed: 11715016]
56. Motoyama N, Naka K. DNA damage tumor suppressor genes and genomic instability. *Curr Opin Genet Dev.* 2004; 14:11–16. [PubMed: 15108799]
57. Osborn AJ, Elledge SJ. Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. *Genes Dev.* 2003; 17:1755–1767. [PubMed: 12865299]
58. Branzei D, Foiani M. The DNA damage response during DNA replication. *Curr Opin Cell Biol.* 2005; 17:568–575. [PubMed: 16226452]
59. Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature.* 2003; 424:1078–1083. [PubMed: 12944972]
60. Hodgson B, Calzada A, Labib K. Mrc1 and Tof1 regulate DNA replication forks in different ways during normal S phase. *Mol Biol Cell.* 2007; 18:3894–3902. [PubMed: 17652453]
61. Tourriere H, Versini G, Cordon-Preciado V, Alabert C, Pasero P. Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. *Mol Cell.* 2005; 19:699–706. [PubMed: 16137625]
62. Petermann E, Helleday T, Caldecott KW. Claspin promotes normal replication fork rates in human cells. *Mol Biol Cell.* 2008; 19:2373–2378. [PubMed: 18353973]
63. Unsal-Kacmaz K, Chastain PD, Qu PP, Minoo P, Cordeiro-Stone M, Sancar A, Kaufmann WK. The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement. *Mol Cell Biol.* 2007; 27:3131–3142. [PubMed: 17296725]

64. Yang XH, Shiotani B, Classon M, Zou L. Chk1 and Claspin potentiate PCNA ubiquitination. *Genes Dev.* 2008; 22:1147–1152. [PubMed: 18451105]
65. Owen BA, Yang Z, Lai M, Gajek M, Badger JD 2nd, Hayes JJ, Edelmann W, Kucherlapati R, Wilson TM, McMurray CT. (CAG)(n)-hairpin DNA binds to Msh2-Msh3 and changes properties of mismatch recognition. *Nat Struct Mol Biol.* 2005; 12:663–670. [PubMed: 16025128]
66. Weaver DT, DePamphilis ML. The role of palindromic and non-palindromic sequences in arresting DNA synthesis in vitro and in vivo. *J Mol Biol.* 1984; 180:961–986. [PubMed: 6098692]
67. Usdin K, Woodford KJ. CGG repeats associated with DNA instability and chromosome fragility form structures that block DNA synthesis in vitro. *Nucleic Acids Res.* 1995; 23:4202–4209. [PubMed: 7479085]
68. Samadashwily GM, Raca G, Mirkin SM. Trinucleotide repeats affect DNA replication in vivo. *Nat Genet.* 1997; 17:298–304. [PubMed: 9354793]
69. Pelletier R, Krasilnikova MM, Samadashwily GM, Lahue R, Mirkin SM. Replication and expansion of trinucleotide repeats in yeast. *Mol Cell Biol.* 2003; 23:1349–1357. [PubMed: 12556494]
70. Voineagu I, Surka C, Shishkin A, Krasilnikova M, Mirkin S. Replisome stalling and stabilization at fragile CGG repeats. *Nat Struct Molec Biol.* 2009 in press.
71. Krasilnikova MM, Mirkin SM. Replication stalling at Friedreich's ataxia (GAA)n repeats in vivo. *Mol Cell Biol.* 2004; 24:2286–2295. [PubMed: 14993268]
72. Sogo JM, Lopes M, Foiani M. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science.* 2002; 297:599–602. [PubMed: 12142537]
73. Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev.* 2005; 19:1040–1052. [PubMed: 15833913]
74. Lambert S, Mason SJ, Barber LJ, Hartley JA, Pearce JA, Carr AM, McHugh PJ. *Schizosaccharomyces pombe* checkpoint response to DNA interstrand cross-links. *Mol Cell Biol.* 2003; 23:4728–4737. [PubMed: 12808110]
75. Lambert S, Watson A, Sheedy DM, Martin B, Carr AM. Gross chromosomal rearrangements and elevated recombination at an inducible site-specific replication fork barrier. *Cell.* 2005; 121:689–702. [PubMed: 15935756]
76. DePamphilis ML, Wassarman PM. Replication of eukaryotic chromosomes: a close-up of the replication fork. *Annu Rev Biochem.* 1980; 49:627–666. [PubMed: 6250448]
77. Svoboda DL, Vos JM. Differential replication of a single, UV-induced lesion in the leading or lagging strand by a human cell extract: fork uncoupling or gap formation. *Proc Natl Acad Sci U S A.* 1995; 92:11975–11979. [PubMed: 8618826]
78. McInerney P, O'Donnell M. Functional uncoupling of twin polymerases: mechanism of polymerase dissociation from a lagging-strand block. *J Biol Chem.* 2004; 279:21543–21551. [PubMed: 15014081]
79. Courcelle J, Donaldson JR, Chow KH, Courcelle CT. DNA damage-induced replication fork regression and processing in *Escherichia coli*. *Science.* 2003; 299:1064–1067. [PubMed: 12543983]
80. Fouche N, Ozgur S, Roy D, Griffith JD. Replication fork regression in repetitive DNAs. *Nucleic Acids Res.* 2006; 34:6044–6050. [PubMed: 17071963]
81. Long DT, Kreuzer KN. Regression supports two mechanisms of fork processing in phage T4. *Proc Natl Acad Sci U S A.* 2008; 105:6852–6857. [PubMed: 18456838]
82. Kerrest A, Anand R, Sundararajan R, Bermejo R, Liberi G, Dujon B, Freudenreich C, Richard G. Srs2 and Sgs1 prevent chromosomal breaks and stabilize triplet repeats by restraining recombination. *Nat Str Molec Biol.* 2009 in press.
83. Balakumaran BS, Freudenreich CH, Zakian VA. CGG/CCG repeats exhibit orientation-dependent instability and orientation-independent fragility in *Saccharomyces cerevisiae*. *Hum Mol Genet.* 2000; 9:93–100. [PubMed: 10587583]
84. Kim H, Narayanan V, Mieczkowski P, Petes Thomas D, Krasilnikova M, SM M, KS L. Chromosome fragility at GAA tracts in yeast depends on repeat orientation and requires mismatch repair. *EMBO Journal.* 2008

85. Lahiri M, Gustafson TL, Majors ER, Freudenreich CH. Expanded CAG repeats activate the DNA damage checkpoint pathway. *Mol Cell*. 2004; 15:287–293. [PubMed: 15260979]
86. Freudenreich CH, Lahiri M. Structure-forming CAG/CTG repeat sequences are sensitive to breakage in the absence of Mrc1 checkpoint function and S-phase checkpoint signaling: implications for trinucleotide repeat expansion diseases. *Cell Cycle*. 2004; 3:1370–1374. [PubMed: 15483399]
87. Lemoine FJ, Degtyareva NP, Lobachev K, Petes TD. Chromosomal translocations in yeast induced by low levels of DNA polymerase a model for chromosome fragile sites. *Cell*. 2005; 120:587–598. [PubMed: 15766523]
88. Lobachev KS, Gordenin DA, Resnick MA. The Mre11 complex is required for repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements. *Cell*. 2002; 108:183–193. [PubMed: 11832209]
89. Raveendranathan M, Chattopadhyay S, Bolon YT, Haworth J, Clarke DJ, Bielinsky AK. Genome-wide replication profiles of S-phase checkpoint mutants reveal fragile sites in yeast. *Embo J*. 2006; 25:3627–3639. [PubMed: 16888628]
90. Razidlo DF, Lahue RS. Mrc1, Tof1 and Csm3 inhibit CAG/CTG repeat instability by at least two mechanisms. *DNA Repair (Amst)*. 2008; 7:633–640. [PubMed: 18321795]
91. Entezam A, Usdin K. ATR protects the genome against CGG/CCG-repeat expansion in Fragile X premutation mice. *Nucleic Acids Res*. 2008; 36:1050–1056. [PubMed: 18160412]

ABBREVIATIONS

ADS	alternative secondary DNA structure
ATM	ataxia and telangiectasia mutated
ATR	ATM and Rad3 related
CHK1 and CHK2	checkpoint kinases 1 and 2
DSB	double stranded break
dsDNA	double stranded DNA
PCNA	proliferating cell nuclear antigen
RPA	replication protein A
SSB	single stranded DNA binding protein
ssDNA	single stranded DNA
TopBP1	topoisomerase binding protein 1
UV	ultraviolet light

CAG/CTG CGG/CCG IRs	IRs	PolyR/PolyY GAA	Telomeric repeats CGG

Figure 1.

Alternative secondary structures and the DNA sequences prone to adopt each type of structure. From left to right: DNA-hairpin, DNA-cruciform, triplex DNA (H-DNA) and quadruplex DNA. Black rectangles - G quartets.

Figure 2.

a. Schematic representation of checkpoint signaling at stalled replication forks. Mec1 is activated by ssDNA bound by RPA and the 9-1-1 complex. Activated Mec1 phosphorylates Mrc1, which recruits Rad53. Mec1-dependent Rad53 activation leads to replisome stabilization, inhibition of origin firing and cell cycle arrest.

● - DNA polymerases, ○ - PCNA, ★ - template lesion arresting the leading strand polymerase, ○ - MCM2-7 helicase unwinding the template ahead of the fork, ● - RPA bound to ssDNA, ● - RFC-like complex, ○ - 9-1-1 complex.

b. Model for the interplay between ADS and checkpoint responses at CGG repeats (left) and CAG repeats (right) leading to frequent chromosomal breakage at CGG repeats and the prevention of chromosomal breakage at CAG repeats (see text for details)